FAX

Urgent

From

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To

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Comment

Attn: Abigail Fisher
RE: US Pat. Appl. No. 10599740
Applicant: Dale Devore

EXAMPLE

Laboratory study to determine the appropriate buffer solution concentration, and pH, and glutaric anhydride concentrations for treating tissues to increase net negative charge.

As noted in the literature (RL Lundblad, "Chemical Reagents for Protein Modification" 2nd edition, CRC Press, 2000), acylation of amino groups on proteins is dependent on pH. The reactive pH range is generally between about 8.0 and 9.0.

This experiment was conducted to determine instant pH (within 5 seconds) and final pH (> 2minutes) after dissolving different concentrations of glutaric anhydride powder in different molality concentrations of sodium phosphate solutions. Buffer compositions must provide an instant reaction pH of between 8.0 and about 8.5 after dissolving acylation agent (5 seconds) and the final pH must be physiologic-between 6.8-7.6. A pH of 8.0 to 8.5 or higher is needed to provide an effective pH to maintain deprotonated amines and allow reaction with selected acylation agents at specific concentrations.

Sodium phosphate solutions at 0.02M, 0.1M, 0.2M, 0.3M, 0.4M, and 0.5M were prepared using monobasic sodium phosphate, monohydrate (Acros) and dibasic sodium phosphate, anhydrous (JT Baker). Solution pH ranged from 7.5-9.15.

Glutaric anhydride (Aldrich Batch 01012JH) was dissolved in sodium phosphate solution at concentrations ranging from 50mg/ml to 2 mg/ml.

Two ml of sodium phosphate solution were pipeted into a 15ml centrifuge tube and the pH measured using the Orion Model 601N pH meter. The pH probe was removed from the centrifuge tube and pre-weighed glutaric anhydride (GA) was rapidly dissolved in 2ml of sodium phosphate solutions (within 5 seconds). The pH probe was immediately placed in the mixture to record the instant pH. The final pH was recorded at least 2 minutes later.

Results are shown in Table 1.

Table 1. pH values of various sodium phosphate solutions before and after glutaric anhydride dissolution

Solution ($NaPO_4$)		GA Concentration (mg/ml)									
		50		30		10		5		2/3	
M	pH	pH 15s	pH 2m	pH 15s	pH 2m	pH 15s	pH 2m	pH 15s	pH 2m	pH 15s	pH 2m
0.02	7.5	*5.2*	*4.25*					*6.97*	*5.00*	*7.2*	*5.4*
0.1	8.5			*7.0*	*4.40*	*7.5*	*6.5*	**8.10**	**7.20**	**8.20(2)** **8.00(3)**	**7.4(2)** **7.10(3)**
0.2	8.8			*7.2*	*5.40*			**8.20**	**7.20**	**8.40**	**7.60**
0.3	9.15			*7.6*	*6.50*	*7.8*	*7.2*	**8.5**	**7.4**	**8.53(3)** **8.70(2)**	**7.6(3)** 7.90(2)
0.4/0/1	8.66					*7.90*	*6.90*	**8.30**	**7.15**		
0.5/0.1	8.58			*7.37*	*6.60*			**8.0**	**7.6**		

As shown, sodium phosphate solution at 0.02M was unable to provide an instant reactive pH between 8.0-8.5, at any GA concentration, including 2mg/ml. All final pH values were acidic and out of the generally accepted physiologic range. GA concentrations greater than 10mg/ml at any tested sodium phosphate concentration did not provide an instant reactive pH greater than 8.0, the low end for acylation reactivity. This was due to the instantaneous hydrolysis of the agents to an acid form, reducing the solution pH below the reactive range of 8.0-9.0. GA at 10mg/ml in 0.3M sodium phosphate did provide an instant pH of 8.0. The acceptable effective ranges for sodium phosphate solution molality and GA concentration were preferably 0.1M to 0.5M and 2mg/ml to 5mg/ml respectively and most preferably 0.2M to 0.3M and 5mg/ml or less (**noted in bold**). Solution molality less than 0.1M was not able to maintain effective reaction pH and GA concentrations greater than 5mg/ml generally resulted in a rapid decrease in pH, lower than the effective reaction pH ranges (due to hydrolysis of GA to glutaric acid).

Based on this investigation, it is concluded that the effective molality of the sodium phosphate solution is from 0.1M to 0.5M, preferably between 0.2M and 0.3M and the effective glutaric anhydride concentration is less than 5mg/ml.